Informatics Analysis of Genetic Variants in Newborn Screening

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Introduction

Newborn screening (NBS) is a well-known and widely accepted activity in public health. The majority of babies born in the United States are screened via a biochemical blood test for conditions that are difficult to diagnose but require treatment soon after birth[1]. NBS began in the 1960s as a blood test for phenylketonuria (PKU) and additional disorders were added to the screening panel as technological advancements, such as tandem mass spectrometry (MS/MS), improved detection of many metabolic disorders[2]. Based on the guidelines set forth by the American College of Medical Genetics, the U.S. Department of Health Secretary's Advisory Committee on Heritable Disorders in Newborns and Children selects disorders for the recommended uniform screening panel (RUSP) if (1) they can be detected 24-48 hours after birth and would not otherwise be detected, (2) there is reliable screening method with appropriate sensitivity and specificity and (3) there are benefits of early detection and treatment[3].

NBS has been successful; however, the technology used in medicine is quickly advancing and NBS programs are faced with the challenge of incorporating new technology into their screening protocol to improve detection of disorders[4]. Next generation sequencing (NGS), including whole exome sequencing (WES), is one of these new technologies. WES identifies the DNA sequence of the coding region of the genome, where it is believed 85% of disease causing genetic variation occurs[5]. With its relatively low cost and ability to provide detailed genetic information, WES is quickly becoming a tool with the potential to revolutionize public health, however, the impact of its use in the clinic for the benefit on overall health remains unknown[6].

There are many potential benefits of incorporating WES into NBS as a majority of the disorders currently screened for are caused by rare recessive genetic variants. By identifying the genetic factors causing the disorder, a physician can tailor treatment to the specific needs of the patient. For example, at least 400 mutations in the *PAH* gene lead to PKU and there are different treatments for the disorder depending on the type of mutation[7]. In addition to using WES to identify the presence of disorders currently screened for, it could be used to screen for other conditions that fit the RUSP criteria but currently lack a screening method. Adding new disorders to the screening panel, however, would require careful consideration of the long-term ethical and psychological effects of a diagnosis as well as the degree to which the disorder is medically actionable[8].

Three challenges must be addressed before NGS can be incorporated into NBS. First, the ability of NGS to detect disorders screened for in NBS must be compared to the current method. Second, additional disorders that cannot be detected by traditional screening methods, but can be detected through NGS, should be investigated for potential addition to NBS. Lastly, if NGS is incorporated into NBS and additional disorders are added to the screening panel, information about NGS should be provided to parents to help them make informed choices to understand the implications of their decisions.[9]

To address the first challenge, the usefulness of WES as a screening tool must be studied to see how well it identifies the presence of disorders currently screened for. The clinical use of WES as a diagnostic tool to identify causal variants for rare diseases has been successful[10] however the effectiveness of WES as a screening tool for RUSP

disorders must be analyzed. In other words, WES has been useful when a patient has symptoms that suggest a link to a genetic variant, but its ability to identify conditions when applied to the general population must be investigated. The presence of a variant in a gene associated with a disorder is not enough to indicate that an individual has the disorder. There are silent and synonymous variants that do not affect gene function as well as variants of uncertain significance (VUS) that have unknown effects on phenotype. In the development of a genetic screening test, it is crucial that the test be able to detect the presence of a condition but not return many false positives. Which variants best indicate a positive test result must be determined, and many variables must be considered, such as (1) the type, location and frequency of the variant, (2) whether the variant is known to be pathogenic or not and (3) the consequences of providing a false positive for the associated condition.

The selection criteria for which genetic variants are expected to indicate a positive test result in NBS must be analyzed for each gene associated with a RUSP condition to create a screening test with a high positive predictive value (PPV). The PPV of a screening test is the proportion of positive results that are true positives and it is calculated from the sensitivity and the specificity of the test as well as the prevalence of the condition screened for[11]. Sensitivity is how well a screening test identifies those who have the condition (true positive rate), and specificity is how well it identifies those without the condition (true negative rate)[12].

To analyze how well WES could perform in NBS, variant selection algorithms (VSA) of increasing sensitivity will be used to screen for genetic variants in 23 genes known to cause 25 of the RUSP disorders. The VSAs will simulate WES screening tests

that require different types of variants to indicate a positive screen. As the VSAs include more possible types of variants, the predicted sensitivity of the VSA increases because the criteria necessary for a positive screen becomes more lenient thus increasing the possibility of identifying all disease causing variants. The VSAs will be applied to the exome sequence data of a population not expected to have any of the RUSP disorders. Thus, any variant detected by the algorithms will be a false positive. For each gene, the specificity and the false positive rate (1-specificity) will be calculated for every VSA based on the number of positive screens returned. The false positive rate will then be compared to the sensitivity of the VSA in a receiver operating characteristic (ROC) curve to estimate the PPV of the VSA in each gene.

ROC curves allow one to tailor the sensitivity cutoff for a screening test to ensure that high sensitivity does not sacrifice specificity[13]. This is particularly important in NBS, since a test with high sensitivity and low specificity would identify all disease causing variants but would return too many false positives. A screening test with low sensitivity and high specificity would not return many false positives but would miss too many disease causing variants, in other words, it would return false negatives. The ROC curves for each gene will be analyzed and used to tailor the appropriate of screening tests for each gene depending on the downstream clinical implications of a positive test result.

Method

Exome Sequences

Two rounds of VSAs, each with increasing sensitivity, were run on a database of exome sequence variants of 619 individuals. This exome sequence data was obtained

from the North Carolina Clinical Genomic Evaluation by Next-gen Exome Sequencing (NCGENES) project that conducted WES on individuals with conditions such as cancer, cardiogenetic diseases, neurodevelopmental disorders, and retinal diseases[14].

The annotated variants from the NCGENES exome sequences were accessed through a Postgres-SQL database[15]. Variants accessed from the database were annotated with the type of mutation, National Center for Biotechnology Information ClinVar database[16] classification, Human Genome Mutation Database[17] (HGMD) classification, conserved functional domain from the RefSeq database[18] and the maximum allele frequency from the Exome Aggregation Consortium (EXAC)[19].

Prevalence of the Conditions

The prevalences of the 25 selected RUSP disorders[20] were estimated based on the overall prevalence from the study "Birth prevalence of disorders detectable through newborn screening by race/ethnicity" [21] which investigated the prevalence of these disorders in newborns in California. The prevalences from this study were useful, because California is a good representation of the general population as it is a large state with a diverse population. The prevalences of each condition were rounded down and the associated genes were placed in groups according to those prevalences (see Appendix I). The associated genes were grouped as prevalences of $\frac{1}{10{,}000}$, $\frac{1}{100{,}000}$, and $\frac{1}{1{,}000{,}000}$. The expected allele frequency for each prevalence group was determined using the Hardy Weinberg Equilibrium equation. Knowing allele frequency is important, because a genetic variant suspected to cause disease should not be at an EXAC maximum allele frequency higher than the allele frequency calculated from the

prevalence of disease. If a variant has a maximum allele frequency higher than the calculated allele frequency for the disorder then the variant is believed be more common than the disease-causing variant.

Table. 1 The prevalence groupings and calculated frequency for each gene.

Prevalence	Frequency Limit	Genes
1/10,000	0.01*	*HBB*
1/100,000	0.003	*ACADM, ACADVL, BTD, GALT, MUT, PAH, SLC22A5, MCCC1, MCCC2, MMAA, MMAB*
1/1,000,000	0.001	*CBS, HMGCL, IVD, ACAT1, ASL, HADHA, HADHB, HLCS, PCCA, PCCB, FAH*

*Variants in *HBB* are more common in African populations

Variant Selection Algorithms (VSAs)

Since this study screened for recessive conditions, an individual must have two variants in the same gene that meet the criteria specified by the VSA in order to return a positive result. The VSAs also only selected variants if the EXAC maximum allele frequency was below the calculated allele frequency for the prevalence group of the associated condition. Five VSAs were used in the first round(Table 2). The VSAs increase in sensitivity with VSA 1 as the least sensitive and VSA 5 as the most sensitive. For example, VSA1 is the least sensitive because it only allows variants that are labeled ‘pathogenic’ to count as a positive screen. VSA 2 increases in sensitivity as it allows the types of mutations that are often disease causing to count as a positive screen but may not be labeled ‘pathogenic’. Although it may seem reasonable to only screen for variants that are known to be pathogenic, a screening test must also be able to identify novel variants that would cause a RUSP disorder. The VSAs are also

additive, so VSA 2 includes all variants that matched the selection criteria for VSA 2 as well as the selection criteria for VSA 1.

Table 2: **The first round of VSAs.** VSAs increase in sensitivity with from VSA 1 – 5

VSA	Selection Criteria
VSA 1	ClinVar assertion is 'pathogenic'
VSA 2	Nonsense, stop-loss, splice site, splice site UTR, frame-shifting indel, nonsense indel
VSA 3	ClinVar assertion 'likely pathogenic' or is labeled disease causing mutation ('DM') in the Human Gene Mutation Database (HGMD).
VSA 4	Missense, boundary crossing indels, potential RNA editing site and non frameshifting indels with CADD[22]scores greater than 13
VSA 5	All rare missense variants

The first round of VSAs did not return many variants. Having the same selection parameters as VSAs used in a study investigating the use of genome screening to detect dominant disorders[23] is likely a contributing factor to why there were so few positive results. Dominant disorders only require one variant to return a positive screen whereas recessive disorders require two variants to return a positive screen. A second round of VSAs with a broader range of sensitivity was created to determine the level of sensitivity that reduced the specificity of the VSAs. VSAs 7-11 in the second round had high sensitivity because they included variants in the selection criteria that are not often disease causing, such as variants in the untranslated region (UTR) and introns, both of which are non-coding regions. Once again the VSAs increase in sensitivity with VSA 1 as the least sensitive and VSA 11 as the most sensitive.

Table 3. The second round of VSAs. VSAs increase in sensitivity with from VSA 1-11

VSA	Selection Criteria
VSA 1	ClinVar pathogenic or likely pathogenic
VSA 2	Truncating (nonsense, frame-shift, canonical splice site, start codon mutation)
VSA 3	Missense and in-frame indels with CADD > 13
VSA 4	Missense and in-frame indels in conserved domains
VSA 5	Missense and in-frame indels
VSA 6	Labeled disease mutations ('DM') in HGMD
VSA 7	Near splice site (+/-3 to +/- 10 in intron)
VSA 8	Synonymous
VSA 9	UTR
VSA 10	Deep intronic variants
VSA 11	Rare

Receiver Operating Characteristic (ROC) Curve Analysis

The specificity of the individual VSAs for each gene was calculated using the number of false positives detected.

Specificity = (True Negatives– False Positives) / (True Negatives)

For both rounds, the VSAs, with increasing sensitivity (true positive rate), were plotted against the false positive rate (1 – specificity) to produce a curve similar to a ROC curve for each gene. ROC curves compare the sensitivity and false positive rate of a screening test and are used to estimate the predictive strength of a test by calculating the area under the curve[24]. More area under the curve indicates a better predictive

value of the test. The curves for each gene can be used to assign a sensitivity cutoff that provides a test with the highest predictive value.

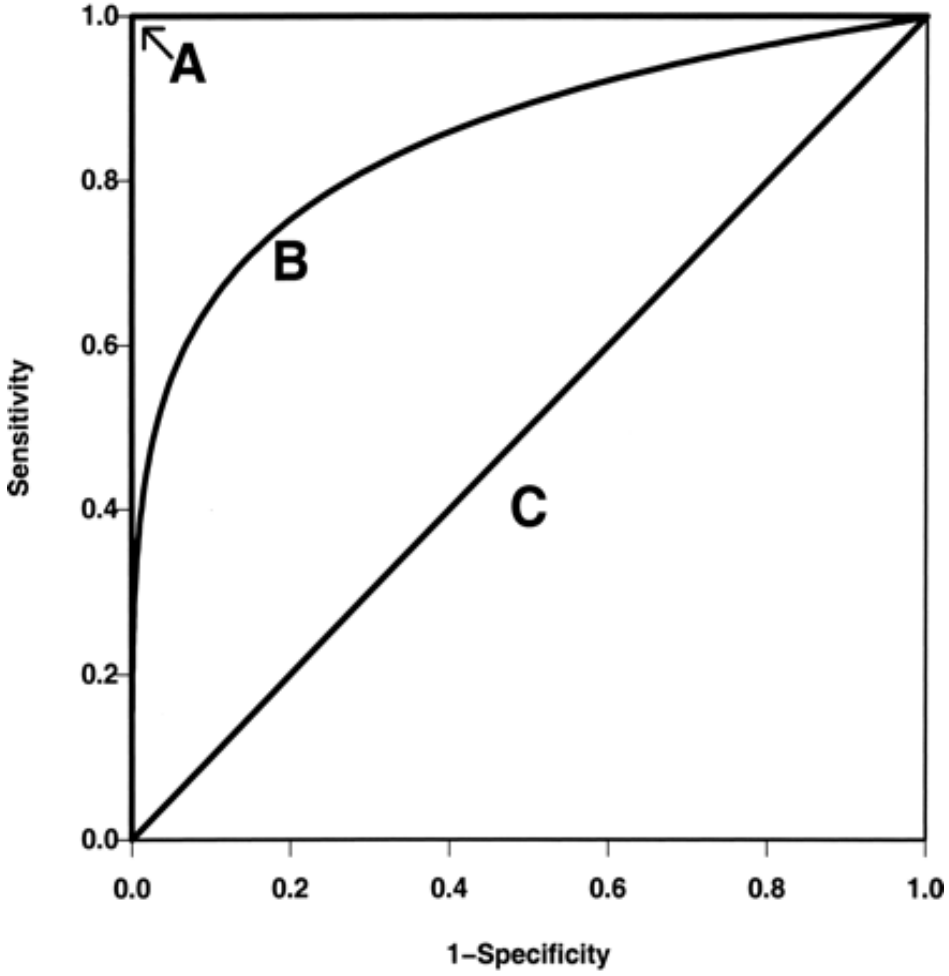

Fig. 1
A model Receiver Operating Characteristic Curve (ROC)[25]. Sensitivity (true positive rate) is plotted on the y-axis against 1-specificty (false positive rate) on the x-axis. The predictive strength of a screening test can be estimated by calculating the area under the curve. A is the screening test with the strongest predictive power with no false positives detected at the highest sensitivity and the area under the curve equal to 1. C is the screening test with poorest predictive power with the area under the curve equal to 0.5.

Results

Two rounds of variant selection algorithms were applied to the NCGENES database. It was assumed that the participants in NGGENES did not have any of the disorders screened for in NBS. Therefore, any variant identified by a VSA in this population would be considered a false positive. Curves were created for the genes associated with the disorders screened for in NBS. The VSAs, in increasing sensitivity, were plotted against the false positive rate. Curves were created for both round of VSAs applied.

The first round of VSAs returned fewer positive results than the second round of more sensitive VSAs overall. For the gene *BTD*, both rounds of VSAs returned positive results. Actually, the first round of VSAs returned positive results only for *BTD* in VSAs 2-5 (Fig.2B), and did not return positive results for any other genes (Fig.2A).

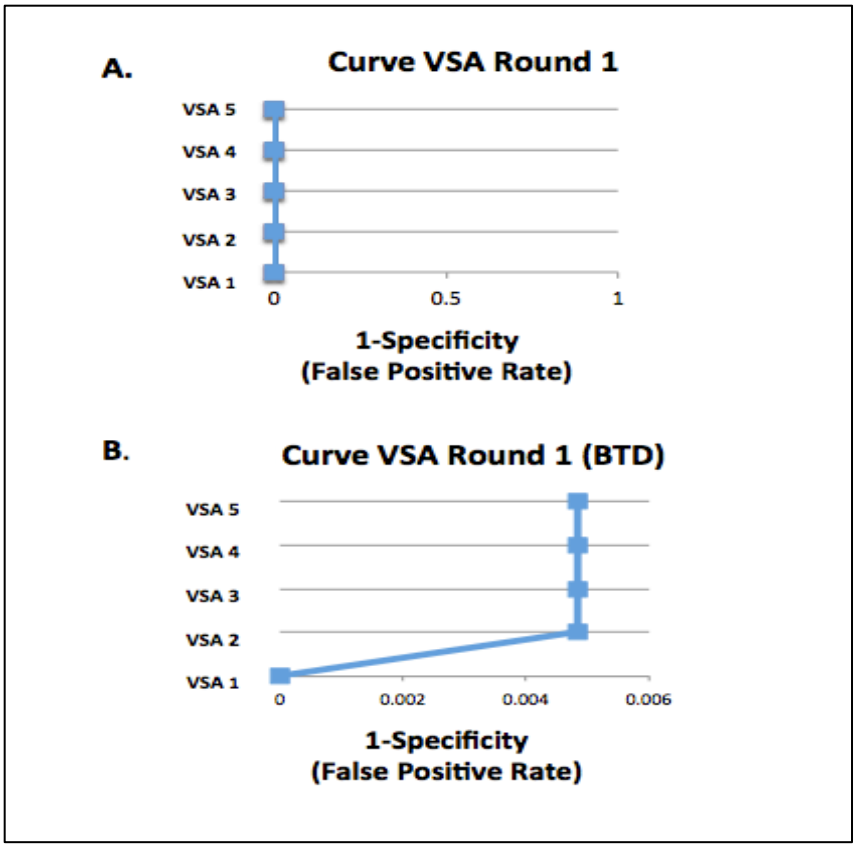

Fig. 2 Curves for the first round of VSAs applied to each gene. The VSAs increase in sensitivity from VSA 1 as the least sensitive to VSA 5 as the most sensitive. The VSAs are plotted against the corresponding false positive rate. (A) The curve for all genes except *BTD*. None of these genes returned positive results. (B) The curve for *BTD.* VSAs 2-5 for *BTD* returned three positive screens.

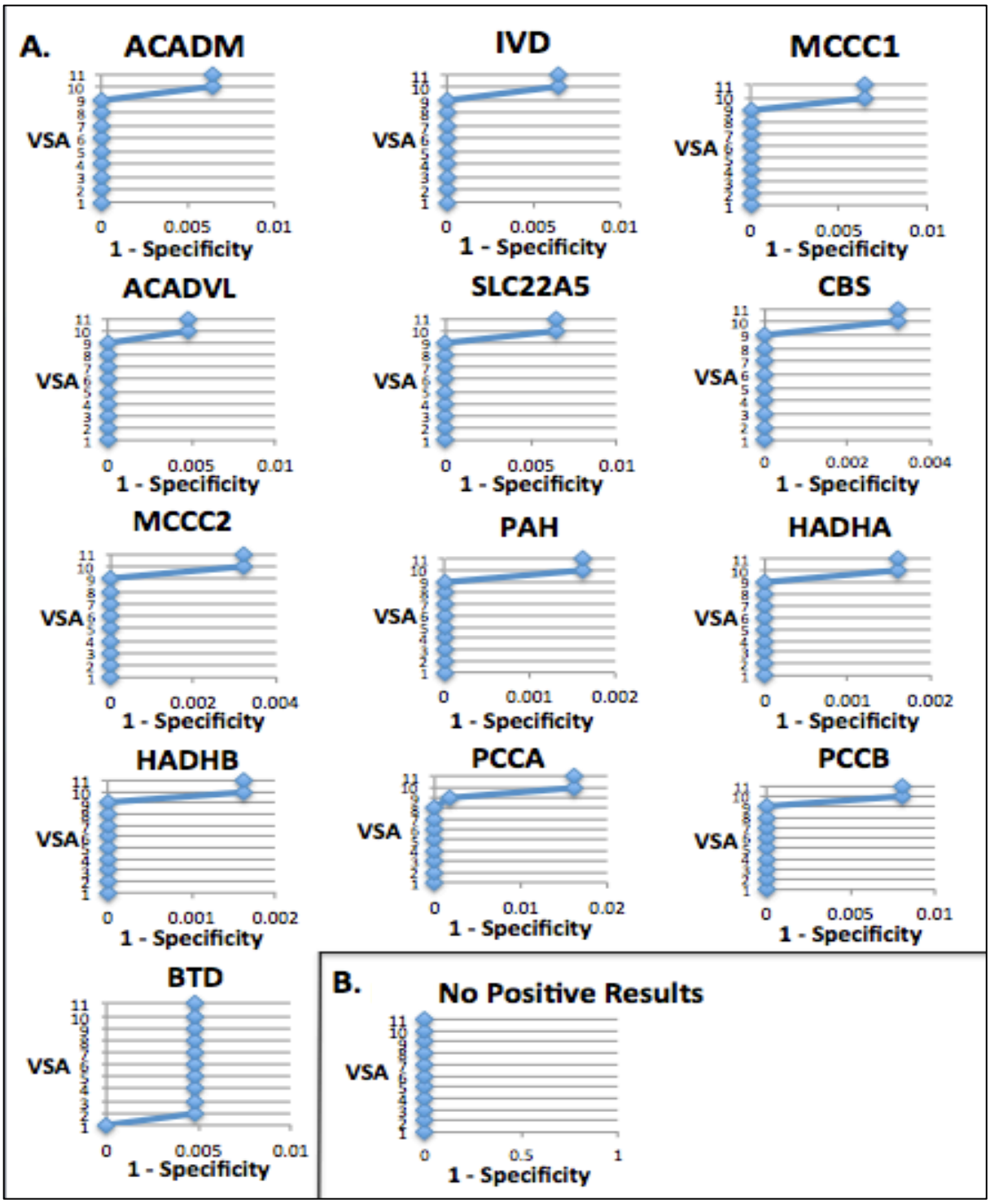

Fig 3. Curves for the second round of VSAs. The VSAs increase in sensitivity from VSA 1 as the least sensitive to VSA 11 as the most sensitive. The VSAs are plotted against the corresponding false positive rate (1-specificity). (A) Individual curves for genes that returned positive results and (B) a general curve for genes that did not return positive results. The genes that did not return positive results are *HHB, GALT, MUT, MMAA, MMAB, HMGCL, HLCS, ACAT1, ASL,* AND *FAH.*

The second round of VSAs (Fig. 3) returned the most positive results (Fig. 3 A) in VSA10 and VSA11, which screened for deep intronic variants and any rare variant, respectively. Most genes did not have any positive results for the other VSAs besides VSA10 and VSA11. The exceptions were *BTD*, which had positive results for VSAs 2-11 and *PCAA*, which had positive results for VSAs 9-11.

Table 4. Positive screen results for round 2 VSAs 1-9. The genes *BTD* and *PCCA* were the only genes to return positive results in any of the VSAs besides VSA10 and VSA 11. These results are shown below. The VSA when the pair of variants was first detected is listed as well as the number of times the pair was detected in that VSA.

VSA 1st Detected	Number of Occurrences	Gene	Chromosome	Position	Type	Ref Allele	Alt Allele
VSA 2	3	*BTD*	3	15676989	frameshifting indel	TG	-
		BTD	3	15676986	frameshifting indel	GG	-
VSA 9	1	*PCCA*	13	100741464	synonymous	G	T
		PCCA	13	101182477	UTR-3	TCAC	-

The gene *BTD* is associated with biotinidase deficiency and the gene *PCCA* is associated with propionic acidemia. The variants were not found on the EXAC database and are likely to be rare non-pathogenic variants. Although none of the detected variants were found in the database, a pathogenic missense variant (rs119103232) G to A substitution was found to occur at the same location as the detected variant on chromosome 3 position 15676986 in the gene *BTD*[26]. There is a mild and severe form of biotinidase deficiency (Appendix I) and it is possible that the detected variants are possibly associated with the mild form of the disorder. The variants detected in the gene *BTD* could also be hypomorphic and require an additional severe mutation in the other allele for the severe form of biotinidase deficiency.

Discussion

The specificity was high for the most sensitive VSAs in both rounds of VSAs applied. This suggests that a genetic screening test for NBS disorders could have high sensitivity without jeopardizing specificity. The high level of specificity, even for the most sensitive VSAs, may be because the NBS disorders are recessive disorders and two variants in a gene were required to meet the selection criteria in order to indicate a positive result. Additionally, as these are rare disorders, it would be unlikely that someone without the disease would have two rare variants in the same gene due to low allele frequencies.

It is reasonable that VSA10 and VSA 11 in the second round of VSAs returned the most positive screens, as they were the most sensitive VSAs. VSA 10 screened for deep intronic variants and VSA 11 screened for any rare variant. Introns do not code for protein and any variant that is deep in the intron and not near an exon splice site is not likely to affect phenotype. Often, VSA 10 and VSA 11 returned the same amount of positive screens in each VSA, which is also reasonable because there are not many other types of variants besides those already screened for in VSA 1-10.

Future studies could further increase the sensitivity of the VSAs by allowing the frequency requirements to be more lenient and monitoring the effect on specificity. The calculated variant allele frequencies, used as a cutoff for the selected variants, were calculated using the prevalences from the prevalence groupings. In an attempt to reduce the number of variants above the variant allele frequency for a gene, the prevalences were rounded down when grouped. For example, *GALT* is associated with a disorder at a prevalence of 5 out of 100,000 and would be placed in the group 1 out of

100,000. Because the variant allele frequency for all genes in that group was based on the variant allele frequency for a prevalence of 1 out of 100,000, variants in *GALT* detected with maximum allele frequency at the frequency for the prevalence of 5 out of 100,000 but above the group frequency would not be included as a positive result. Additionally, variants had to be at a maximum allele frequency less than the calculated variant frequency, so a variant with maximum allele exactly at the calculated variant frequency would not be included as a positive result.

Issues regarding frequency can be investigated by being more lenient with the variant allele frequencies required. Each VSA could have two versions, one with a strict variant allele frequency and another with a more lenient one. Additionally, the prevalence groupings could be made with smaller ranges between the least prevalent and most prevalent conditions in a group.

Furthermore, some disease causing variants are more common in certain populations than others, as seen in the gene *HBB* associated with sickle cell disease. Although the VSAs were run with the calculated allele frequency from the overall prevalence of sickle cell disease in the general population, it is likely that variants causing sickle cell would not have been detected if the VSAs were run on a population of individuals known to have sickle cell disease. Sickle cell disease is more prevalent in African populations, meaning the variant occurs at a maximum allele frequency in the African population much higher than the calculated variant allele frequency from the overall population. An individual with a variant at the maximum allele frequency for sickle cell in African populations would not be detected by the VSAs because the maximum allele frequency would be too high. The allele frequency requirements of the

VSAs would need to be adjusted if run on a gene associated with a disease more prevalent in certain populations.

Overall, this study was successful at determining the specificity of the VSAs. The created curves can be used as a tool to estimate PPV of using WES to screen for RUSP disorders in each gene; however, specificity alone is not enough to accurately calculate the PPV. The sensitivity of the VSAs is also needed to calculate the PPV and create an effective screening test. Sensitivity will be measured by examining how well the VSAs identify true positives by running the VSAs on a population of individuals with the RUSP conditions. Additionally, although the VSAs are assumed to be increasing in sensitivity, they must be experimentally tested. For example, VSA 6 is thought to be more sensitive than VSA 4, however VSA 6 may actually be less sensitive than VSA 4 and identify less true positives. Experimentally measuring the sensitivity of the VSAs would also reveal whether the VSAs have different orders of sensitivity depending on the gene. VSA 6 may be more sensitive than VSA 4 in one gene but less sensitive than VSA 4 in another.

With further investigation and the calculation of VSA sensitivity, the proper selection criteria for which variants detected through sequencing in genes associated disorders screened for in NBS can be determined based on the specificity and sensitivity. Downstream effects of a positive result, the risks associated with false positives, degrees of how medically actionable a disorder is and whether or not there is a confirmatory blood test must be carefully considered when determining what selection criteria to use as indication of a positive test result if WES is to be incorporated into NBS in the future.

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Appendix

I.

Gene	Disorder	California Prevalence[27] (per 100,000)	Grouping
ACADM	Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)	5.3	1/100,000
ACADVL	Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD)	1.8	1/100,000
BTD	Biotinidase deficiency (BIO) profound	1.7	1/100,000
	Biotinidase deficiency (BIO) partial	2.2	1/100,000
CBS	Homocystinuria (cystathionine beta synthase) (HCY)	0	1/1,000,000
FAH	Tyrosinemia	0.1*	1/1,000,000
GALT	Galactosemia/galactose-1-phosphate uridyltransferase deficiency (GALT)	1.3	1/100,000
	Galactosemia (Duarte)	5.6	1/100,000
HBB	Sickle cell anemia	10.6	1/10,000
	Beta - Thalassemia	0.7	
HMGCL	3-Hydroxy-3-methylglutaryl-CoA lyase deficiency (HMG)	0	1/1,000,000
IVD	Isovaleric acidemia/ Isovaleryl-Co-A dehydrogenase deficiency (IVA)	0.9	1/1,000,000
MUT	Methylmalonic aciduria (MMA)	.8 - 1.4	1/100,000

PAH	Phenylketonuria/Hyperphenylalaninemia (PKU)	2.9	1/100,000
SLC22A5	Carnitine uptake defect/carnitine transport defect (CUD)	1.5	1/100,000
ACAT1	Beta-ketothiolase (BKT)/ Short-chain keto acylthiolase deficiency (SKAT)	0.1	1/1,000,000
ASL	Argininosuccinic aciduria (ASA)	0.2	1/1,000,000
HADHA	Trifunctional protein deficiency (TFP)	0	1/1,000,000
HADHB	Trifunctional protein deficiency (TFP)	0	1/1,000,000
HLCS	Multiple carboxylase deficiency (MCD)	0.1	1/1,000,000
MCCC1	3-methylcrotonyl-CoA carboxylase deficiency (3-MCC)	2.9	1/100,000
MCCC2	3-methylcrotonyl-CoA carboxylase deficiency (3-MCC)	2.9	1/100,000
MMAA	Methylmalonic aciduria (MMA)	.8 - 1.4	1/100,000
MMAB	Methylmalonia aciduria (MMA)	.8 - 1.4	1/100,000
PCCA	Propionic acidemia	0.2	1/1,000,000
PCCB	Propionic acidemia	0.2	1/1,000,000

The RUSP newborn screening disorders and the associated gene and condition prevalence from "*Birth prevalence of disorders detectable through newborn screening by race/ethnicity".* The assigned grouping for each gene is also shown.

*The technology to detect Tyrosinemia came out halfway through the California study so the prevalence is from Gene Reviews[28].